

Histone modifications induced by a family of bacterial toxins

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Upon infection, pathogens reprogram host gene expression. In eukaryotic cells, genetic reprogramming is induced by the concerted activation/repression of transcription factors and various histone modifications that control DNA accessibility in chromatin. We report here that the bacterial pathogen *Listeria monocytogenes* induces a dramatic dephosphorylation of histone H3 as well as a deacetylation of histone H4 during early phases of infection. This effect is mediated by the major listerial toxin listeriolysin O in a pore-forming-independent manner. Strikingly, a similar effect also is observed with other toxins of the same family, such as *Clostridium perfringens* perfringolysin and *Streptococcus pneumoniae* pneumolysin. The decreased levels of histone modifications correlate with a reduced transcriptional activity of a subset of host genes, including key immunity genes. Thus, control of epigenetic regulation emerges here as an unsuspected function shared by several bacterial toxins, highlighting a common strategy used by intracellular and extracellular pathogens to modulate the host response early during infection.

epigenetics | *Listeria* | pathogenesis

One of the best model organisms for the study of host–pathogen interactions is *Listeria monocytogenes*, a facultative intracellular pathogen well known for its sophisticated exploitation of host cell processes and molecular mimicry (1). This Gram-positive bacterium causes foodborne infections that can lead to sepsis and meningitis mostly in immunocompromised patients and to abortion in pregnant women. Although well adapted to survive extracellularly, this pathogen can infect, survive, and replicate in the cytoplasm of both macrophages and nonprofessional phagocytic cells, such as epithelial cells.

Entry of *L. monocytogenes* into the host cytoplasm depends on escape from the phagosome, a process mediated by the key virulence factor listeriolysin O (LLO). LLO is part of a large family of pore-forming toxins, the cholesterol-dependent cytolysins (CDC), expressed by many different unrelated bacteria of different genera (e.g., *Clostridium*, *Streptococcus*, *Listeria*, *Bacillus*, and *Arcanobacterium*) (reviewed in ref. 2). Beyond their pore formation ability, these toxins have evolved other elaborate mechanisms by which they act. In the case of *Listeria*, LLO's optimum low pH lytic activity allows bacteria trapped in phagosomes to lyse and exit these vacuoles without causing damage to the plasma membrane. In addition, LLO is secreted before entry of the bacteria into host cells and functions extracellularly as a potent signaling molecule (3). By mechanisms still not well understood, LLO induces MAPK and calcium signaling in a pore-forming-dependent manner (4–6) and NF- κ B signaling in a pore-forming-independent manner (7).

Cellular responses to pathogenic microorganisms have recently been extensively studied by using DNA microarray techniques. These studies have shown that the host transcriptome undergoes large changes upon infection (8). Transcriptional profiling of macrophages infected with *L. monocytogenes* and *in vivo* studies shows that, upon entry in the cytosol, a large immune response is activated (9, 49).

Gene expression can be controlled by a large number of regulatory proteins. Many coactivators and corepressors also are involved and some catalyze covalent modifications of the DNA-associated histones. Specific combinations of posttranslational modifications at the tails of histone proteins, frequently referred to as the histone code, act in concert to generate, stabilize, or occlude DNA binding sites for regulatory proteins such as transcription factors (10). In fact, histone modifications are necessary to induce a complete transcriptional response (11, 12). Histone modifications such as phosphorylation of Ser¹⁰ on histone H3 and acetylation of lysines on histones H3 and H4 have been documented for being associated with transcriptional activation (13, 14). Interestingly, viruses have mastered manipulation of the histone code, which they use to control DNA accessibility and stability of both cellular and viral genomes (15).

In this article, we demonstrate that *L. monocytogenes*, before invasion, modulates host gene expression through histone modifications. Early in infection, extracellular *L. monocytogenes* induces a drastic dephosphorylation of Ser¹⁰ on H3 and deacetylation of H4 by secreting LLO. These modifications correlate with transcriptional reprogramming of a subset of host genes, including decreased expression of key immunity factors. Strikingly, dephosphorylation of Ser¹⁰ is a feature shared by at least two other toxins of the LLO family, namely *Clostridium perfringens* perfringolysin (PFO) and *Streptococcus pneumoniae* pneumolysin (PLY), revealing a general mechanism of epigenetic regulation used by unrelated bacteria.

Results

***L. monocytogenes* Induces Specific Histone Modifications During Infection.** To determine whether *L. monocytogenes* induced histone modifications during infection, we first focused on phosphorylation of Ser¹⁰ on histone H3. We harvested infected HeLa cells at different time points after the start of infection and measured the levels of modified H3 by Western blotting experiments. Fig. 1 shows that, after a transient 1.5-fold increase in phospho-Ser¹⁰ H3, *L. monocytogenes* induces a marked dephosphorylation of Ser¹⁰ H3. The maximal effect, showing a 4-fold decrease compared with uninfected cells, is observed after 3 h of infection. After 5 h of

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Abbreviations: LLO, listeriolysin O; CDC, cholesterol-dependent cytolysin; PFO, perfringolysin; PLY, pneumolysin; PA, protective antigen; RT, reverse transcription; qPCR, quantitative PCR; acH4, acetyl-H4.

Data deposition: The sequence reported in this paper has been deposited in the MIAMExpress database (accession no. E-MEXP-1078).

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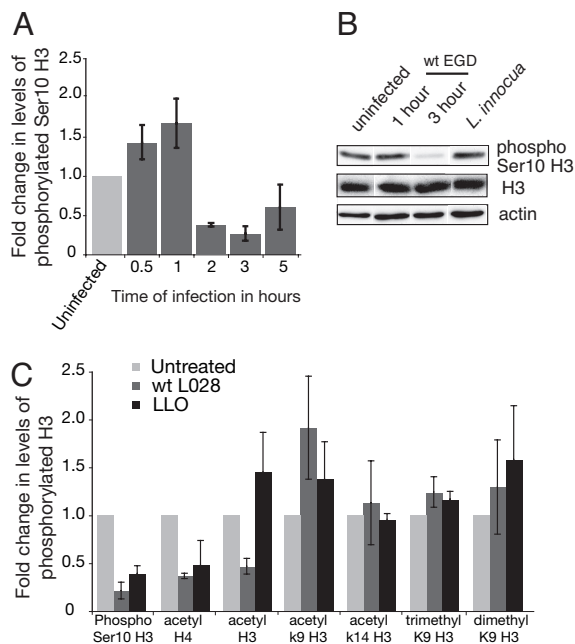


Fig. 1. Histone modifications induced by *L. monocytogenes*. (A) Levels of phospho-Ser¹⁰ H3 as detected by Western blot were quantified and normalized, first to actin levels and then to the uninfected sample. Error bars represent SEM of at least three separate experiments. (B) Immunoblots were performed on uninfected cells and cells infected with wild *L. monocytogenes* and *L. innocua*. (C) Quantification of different histone modifications in uninfected cells, cells infected with wild-type *L. monocytogenes*, and cells treated with 6 nM LLO using antibodies described in *Materials and Methods*. Error bars show the SEM of three independent experiments.

infection, the levels of phospho-Ser¹⁰ H3 increase, although they do not reach the levels observed in uninfected cells (Fig. 1). Importantly, whereas the levels of phospho-Ser¹⁰ H3 are decreased on *L. monocytogenes* infection, the total level of histone H3 does not vary (Fig. 1B), implying that bacteria do not induce degradation of histones but specifically induce dephosphorylation of Ser¹⁰ H3.

To investigate the effect of *L. monocytogenes* on other histone modifications besides phosphorylated Ser¹⁰ H3, we compared the levels of multiple modifications in cells infected for 3 h to noninfected cells. Our results show that, along with dephosphorylating Ser¹⁰ H3, *L. monocytogenes* induces a significant decrease in the levels of acetyl-H3 and acetyl-H4 (acH4) but has no effect on methyl-H3 (Fig. 1C). Therefore, *L. monocytogenes* induces a specific histone response, which includes dephosphorylation and deacetylation of H3 and deacetylation of H4.

Extracellular, Pathogenic *Listeria* Induce Dephosphorylation of Ser¹⁰ H3. Dephosphorylation of Ser¹⁰ H3 was observed early in infection, suggesting that *L. monocytogenes* did not need to enter the cytoplasm of host cells to induce this effect. To test whether invasion of bacteria is required for dephosphorylation of Ser¹⁰ H3, cells were treated with cytochalasin D, an actin polymerization inhibitor that prevents entry of *L. monocytogenes* (16). Fig. 2A shows that *L. monocytogenes* is still able to decrease the levels of phospho-Ser¹⁰ in cytochalasin D-treated cells, revealing that invasion is not required for dephosphorylation of Ser¹⁰ H3.

The closely related *Listeria innocua* species is nonpathogenic and was therefore tested for its ability to induce dephosphorylation of Ser¹⁰ H3. Interestingly, this modification of host histones was not observed (Fig. 1B), and it was observed only upon infection with the pathogenic species *L. monocytogenes*, e.g., a wild-type EGD (Fig. 1B) or L028 (Fig. 2B) strain. Therefore,

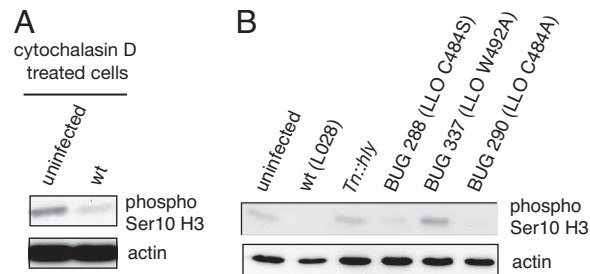


Fig. 2. Dephosphorylation of Ser¹⁰ H3 is induced by extracellular *L. monocytogenes* through LLO. (A) HeLa cells were treated with 5 μ g/ml cytochalasin D for 15 min followed by infection for 3 h. (B) HeLa cells were infected with either wild-type *L. monocytogenes* (L028 strain), a transposon mutant of *hly* (BOF415) (48), or one of three *hly* point mutants (BUG 288, BUG 337, or BUG 290) (21) for 3 h before extraction. All results are representative of at least three independent experiments.

dephosphorylation of Ser¹⁰ is mediated by a factor present in *L. monocytogenes* and absent in *L. innocua*.

LLO Is the Major Factor Inducing Dephosphorylation of Ser¹⁰ and Deacetylation of H4. To identify factors important for inducing dephosphorylation of Ser¹⁰ during infection, we studied the effect of several mutants defective for various virulence factors. A *ΔinlB* mutant defective for an internalization protein important for HeLa cell invasion was first tested and found to have no effect on phospho-Ser¹⁰ H3 levels compared with wild type (data not shown), reinforcing the finding that *L. monocytogenes* entry is not required to induce this effect. Another mutant defective for LLO, *Δhly*, was tested for its ability to dephosphorylate Ser¹⁰ H3. Strikingly, this mutant had no effect on levels of phospho-Ser¹⁰ H3, compared with the wild-type strain (Fig. 2B). Complementation of the mutation restored the wild-type phenotype, strongly suggesting that LLO is the major factor responsible for Ser¹⁰ H3 dephosphorylation.

To then determine whether LLO is sufficient to induce histone modifications, HeLa cells were treated with purified protein. Fig. 1C shows that purified LLO protein does induce dephosphorylation of Ser¹⁰ H3 and deacetylation of H4 but not deacetylation of H3. LLO is therefore the major protein required for these two modifications, and another factor must be required to deacetylate H3.

To characterize the kinetics of Ser¹⁰ H3 dephosphorylation, HeLa cells treated with LLO were harvested at different time points. Fig. 3A shows that dephosphorylation of Ser¹⁰ H3 occurs as early as 5 min after incubation with LLO and is maximal by 20 min. The effect on Ser¹⁰ H3 is thus much more rapid upon incubation with purified protein than with bacteria, which can be explained by the experimental protocol we used. In routine invasion assays, bacteria are thoroughly washed before infection, thereby eliminating any LLO secreted in the medium during exponential growth. Therefore, the delay observed with bacteria versus the purified protein probably reflects the time necessary for the bacteria to produce and secrete a sufficient quantity of LLO to induce dephosphorylation of Ser¹⁰ H3.

To determine whether the observed effect is dose-dependent, HeLa cells were treated with different concentrations of LLO. Fig. 3B shows that 1.2 nM LLO has a negligible effect on phospho-Ser¹⁰ H3, whereas 6 nM induces a 4-fold decrease in phospho-Ser¹⁰ H3. Because 6 nM is the lowest concentration of LLO sufficient to dephosphorylate Ser¹⁰ H3, this concentration was therefore used in further experiments. Importantly, dephosphorylation of H3 is not restricted to HeLa cells and was observed in other cell types, such as LoVo cells and HepG2 cells (data not shown), revealing that our results are not restricted to one cell line.

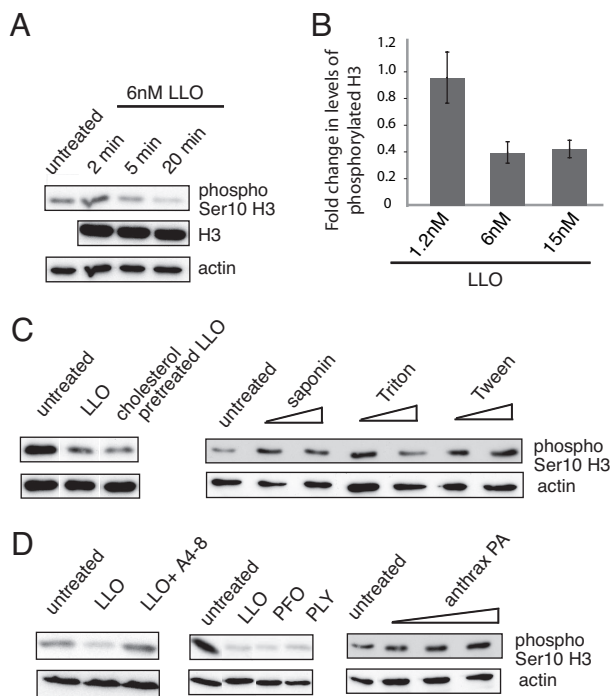


Fig. 3. LLO is the listerial factor required for inducing the decrease in levels of phospho-Ser¹⁰H3. (A) Cells were incubated with 6 nM LLO for the indicated times before extraction and immunoblotting. (B) Cells were incubated with the indicated concentrations of LLO for 20 min before immunoblotting and quantification. Error bars represent the SEM of at least three separate experiments. (C) Six nanomolar LLO pretreated with 20 nM cholesterol for 30 min on ice or with 0.0006% and 0.003% saponin, Triton X-100, or Tween 20 was added to cells for 20 min. (D) Six nanomolar LLO or 6 nM LLO with 13.2 ng of A4-8 neutralizing antibody was incubated for 1 h at 37°C before adding to cells for 20 min. Cells were incubated with PFO and PLY at the same hemolytic titer as LLO for 20 min. Anthrax PA was used at 1.4, 7, and 35 nM, respectively. All results are representative of at least three independent experiments.

LLO Induces Dephosphorylation of Ser¹⁰ H3 Through a Pore-Forming-Independent Mechanism. To understand the mechanism by which LLO dephosphorylates Ser¹⁰ H3, we investigated whether this effect was due to LLO's pore-forming ability or other signaling activities. Neutralization of pore formation without inhibiting LLO binding to the host cell as previously shown can be obtained by preincubation of LLO with cholesterol (17). As shown in Fig. 3C, preincubation of LLO with 20 nM cholesterol before HeLa cell treatment, although neutralizing protein-permeable pore formation, does not affect its ability to dephosphorylate Ser¹⁰ H3 [supporting information (SI) Fig. 5]. Additional experiments were carried out to determine whether nonspecific perforations in the cell membrane could have the same effect on phospho-Ser¹⁰ H3 as does LLO treatment. The detergents saponin, Tween 20, and Triton X-100, which are commonly used to permeabilize cells and cause comparable lactate dehydrogenase release from HeLa cells as LLO (data not shown), cannot induce a similar decrease in phospho-Ser¹⁰ H3 (Fig. 3C). Together, our data therefore argue that large protein-permeable pore formation by LLO is not required for H3 modification.

Further experiments were carried out to determine whether membrane binding by LLO is the important feature for dephosphorylating Ser¹⁰. An antibody, A4-8, previously shown to prevent LLO binding (SI Fig. 5) (18), was incubated with purified LLO before HeLa cell treatment. Fig. 3C shows that pretreatment of LLO with A4-8 prevented the LLO-induced dephosphorylation of Ser¹⁰ H3. Additionally, strains expressing LLO bearing point mutations in the cell binding region, the ECTGLAWEWWR unde-

capeptide conserved in all toxins of this family (19, 20), were tested for their ability to dephosphorylate Ser¹⁰ H3 (Fig. 2B). These mutants are affected differently for their hemolytic activity, where BUG 337 (LLO W492A) is more affected than BUG 288 (LLO C484S), which itself is more affected than BUG 290 (LLO C484A) (21). In perfect correlation with these mutants' reported lytic activity, we observed the same gradient in their capacity to induce dephosphorylation Ser¹⁰ H3. BUG 290 displays a phenotype close to wild type, whereas BUG 337 resembles a Δhly mutant, and BUG 288 has an intermediate effect. These results show that a single point mutation in the cell binding region of LLO is sufficient to block the effect on phospho-Ser¹⁰ H3. Collectively, our experiments with the membrane-binding blocking antibody and LLO mutants indicate that membrane binding is essential for Ser¹⁰ H3 dephosphorylation.

PLY and PFO Also Induce Dephosphorylation of Ser¹⁰ H3. Because LLO is part of the large family of CDC toxins expressed by unrelated bacteria, we hypothesized that dephosphorylation of Ser¹⁰ H3 would not be restricted to LLO and could be a property shared by other members of this family. To test our hypothesis, we treated HeLa cells, in a manner similar to that used for LLO, with purified PFO of *C. perfringens* and PLY of *S. pneumoniae*. Remarkably, both PLY and PFO induce dephosphorylation of Ser¹⁰ H3 to levels comparable with those induced by LLO after 20 min of incubation (Fig. 3C). Furthermore, similarly to LLO, preincubation of PFO and PLY with cholesterol, which blocks pore formation, does not inhibit their effect on phospho-Ser¹⁰ H3 (data not shown). Therefore, PFO and PLY also induce dephosphorylation of Ser¹⁰ H3 through a mechanism analogous to that of LLO, demonstrating that modification of histones is a property shared by at least three members of this toxin family.

To determine whether dephosphorylation of Ser¹⁰ H3 was a result of an active mechanism carried out by these toxins rather than an unspecific response of the cell to insertion of an oligomeric complex at the cell surface, we treated HeLa cells with *Bacillus anthracis* protective antigen (PA). PA inserts into the plasma membrane oligomerizes but does not form pores unless placed under acidic conditions. Fig. 3D shows that, in contrast to LLO, PFO, and PLY, PA does not induce a change in the level of phospho-Ser¹⁰ H3. Therefore, dephosphorylation of Ser¹⁰ H3 is not an unspecific cellular response to the presence of an oligomeric complex on the cell surface; rather, it is induced uniquely by LLO, PFO, and PLY.

LLO Induces a Specific Transcriptional Response. We hypothesized that the drastic effects of LLO on host epigenetics would also be reflected in transcriptional modulation. To address this question, we determined the global transcriptional response of cells incubated with LLO for 20 min by using human genome Affymetrix chips designed to analyze the expression level of >47,000 transcripts. HeLa cells were incubated for 20 min with 6 nM LLO (same conditions as above), and the whole genome profile was compared with the transcriptional profile of untreated cells. The results of three independent experiments were integrated, and genes that were differentially regulated to statistical significance were recorded. Our analysis identified 47 genes repressed by >1.5-fold and 99 genes induced by >1.5-fold after 20 min of LLO incubation (SI Table 1).

To confirm the results obtained by microarray analysis, reverse transcription (RT) followed by quantitative PCR (qPCR) was performed. Ten genes were randomly selected for confirmation, five that were down-regulated by LLO, three that were up-regulated, and two that were unchanged, and all of them showed the same expression pattern by RT-qPCR as by microarray analysis (Fig. 4 and SI Table 1). Therefore, purified LLO induces a specific transcriptional response in the host, modulating the expression of

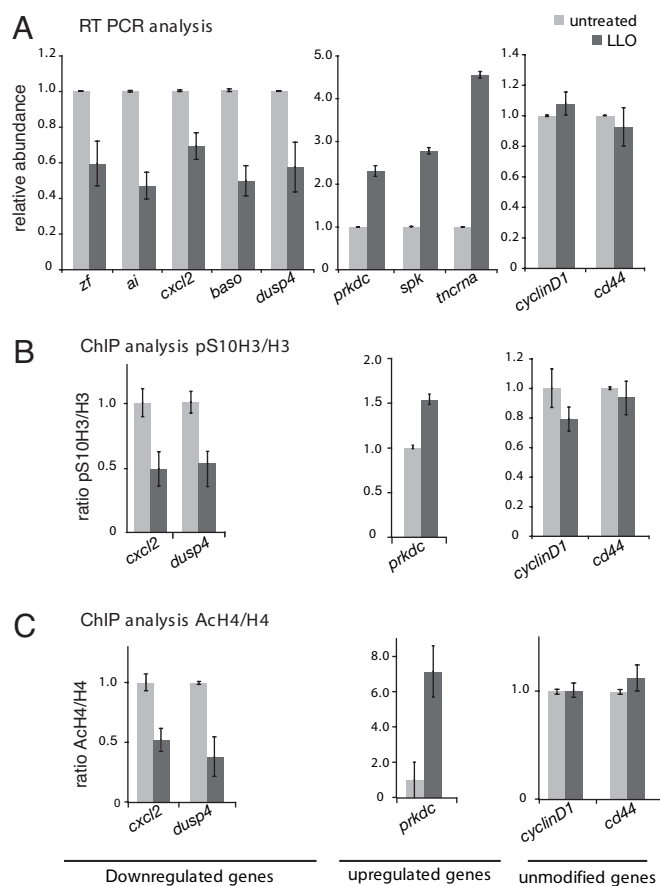


Fig. 4. Changes in levels of transcription induced by LLO correlate with changes in associated modified histones. (A) Quantitative RT-PCR. The mRNA relative abundance of indicated genes is shown normalized to untreated cells and to hypoxanthine phosphoribosyltransferase (HPRT) or 18S as unmodified controls. Data are the average of at least two independent experiments. (B) Antibodies against phospho-Ser¹⁰ H3 (Abcam-12181) and H3 (Abcam-1791) were used to immunoprecipitate chromatin from formaldehyde-fixed HeLa cells untreated or treated with 6 nM LLO for 20 min. Promoter region of the indicated genes were quantified by qPCR. Data are represented as the ratio of phospho-Ser¹⁰ H3/H3 of each enrichment, relative to values obtained with unrelated IgG for each condition. Error bars represent the SEM. (C) Same experiment as in B, except immunoprecipitations were performed with anti-acH4 (Upstate 06-866), and anti-H4 (Abcam-5823).

a small subset of genes as soon as 20 min after the start of incubation.

Modulation of Phosphorylated Ser¹⁰ H3 Levels Occurs Only at Genes Whose Expression Is Regulated by LLO. The transcriptional response of HeLa cells to incubation with LLO identified a subset of genes whose expression is modulated. This raised the question of whether dephosphorylation of Ser¹⁰ H3 is restricted to these differentially expressed genes or whether it is a global phenomenon affecting all genes. To address this issue, we used ChIP to measure the level of phospho-Ser¹⁰ H3 at the promoters of several genes identified as differentially expressed on LLO treatment. As classically performed, the cellular DNA was immunoprecipitated by using antibodies against phospho-Ser¹⁰ H3, or H3, and nonimmune isotype-matched IgG. The immunoprecipitated DNA was analyzed by qPCR to measure the amount of specific chromatin associated to the modified histones and normalized to the amount of chromatin immunoprecipitated with IgG and then to the amount of chromatin associated to H3. These experiments were performed on a selection of genes representing those down-regulated by LLO, those up-

regulated, and those unchanged. Fig. 4 shows that, for example, two genes, *cxcl2* and *dusp4*, identified as down-regulated by microarray analysis, show a decrease in the level of associated phospho-Ser¹⁰ H3 on LLO treatment. In contrast, if the same experiment is performed to analyze *prkdc*, a gene identified as up-regulated by LLO, we observe an increase in the level of associated phospho-Ser¹⁰ H3. If we analyze genes whose expression is unchanged on LLO treatment, such as *cyclinD1* and *CD44*, there is no difference in the level of associated phospho-Ser¹⁰ H3 (Fig. 4B). These data therefore unambiguously indicate a correlation between the expression intensity and the level of associated phospho-Ser¹⁰ H3. More importantly, these ChIP experiments show that the effect LLO exerts on host histones is specific to a subset of genes, those whose expression level is modulated by LLO.

Modulation of Acetylated H4 by LLO Correlates with Ser¹⁰ Modifications. Our Western blots performed on whole-cell lysates indicated that LLO induces a decrease in both H3 phosphorylation and H4 acetylation (Fig. 4). Furthermore, our ChIP analysis indicates that LLO modifies phospho-Ser¹⁰ H3 at a subset of genes. To determine whether modifications of H4 occur at the same subset of genes as those affected on Ser¹⁰ H3, ChIP were performed to detect the levels of acH4. Fig. 4C shows for example that two genes repressed by LLO, *cxcl2* and *dusp4*, have decreased acH4 levels, whereas *prkdc*, whose expression is induced, showed an increase in acH4, and *cyclinD1* and *CD44* show no change in the level of acH4. Therefore, the effect of LLO on acH4, just like its effect on phospho-Ser¹⁰, occurs at a subset of genes, and the same subset of genes appears to be targeted for both H3 and H4 modifications.

Discussion

We report here a mechanism by which, before entry into cells, invasive *L. monocytogenes* manipulate the host gene expression. We demonstrate that the LLO protein secreted by *L. monocytogenes* induces modification of host histones. Epigenetic regulation by LLO is thus a so far unsuspected function for this key virulence factor, known for its pore-forming and potent signaling abilities. The results presented here demonstrate that the effect LLO is having on host histones depends on its ability to bind to the membrane but not on its cytotoxic pore-forming capacity. Our results showing that cell permeabilization by multiple detergents do not induce dephosphorylation of Ser¹⁰ H3 further demonstrate that the observed histone modifications are not an unspecific cellular response to cytotoxic pore formation ability of LLO. Indeed, at the concentration of LLO used in this study, we do not observe any detectable cytotoxicity to cells, because the cell cycle and cellular/nuclear morphology are unchanged on LLO treatment (data not shown). Therefore, our results demonstrate a specific mechanism induced by extracellular LLO. It is unlikely that LLO modifies host histones once inside the cytoplasm because this protein inserts into the plasma membrane and is rapidly degraded in the cytoplasm of the host when bacteria escape from the vacuole (3). Rather, LLO most likely activates a signaling cascade. Activation of different host signaling pathways by LLO has been reported, but the underlying mechanisms remain elusive. Previous studies have shown that LLO induces calcium signaling, the MAPK pathways, protein kinase C, and NF- κ B signaling (4–7, 22). However, treating cells with inhibitors to these pathways {EGTA and BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid] for calcium signaling; SB202190, PD98059, and SP600125 for MAPK signaling (p38, p42/48, and JNK, respectively); and ALL-N and BAY 11-7085 for NF- κ B signaling} did not block the effect on Ser¹⁰ H3 induced by LLO, even though all inhibitors blocked their respective targeted pathway (data not shown). These results are consistent with previous studies showing that calcium, MAPK, and NF- κ B signaling induce an increase in phospho-Ser¹⁰ H3, rather than a decrease as we observe here (23–25). Therefore, dephosphorylation of Ser¹⁰ H3 must be induced through a pathway not yet described as activated

by LLO. Inhibitors to other pathways also were used in an attempt to identify the signaling mechanism induced by LLO. Inhibitors to tyrosine kinases (genistein), serine/threonine kinases (staurosporine), and phosphatidylinositol 3-kinases (LY294002) all had no effect on the induced decrease in phospho-Ser¹⁰ H3, suggesting that none of these pathways is involved in LLO's modulation of host epigenetics.

Remarkably, epigenetic regulation by LLO induces dramatic dephosphorylation of Ser¹⁰ H3. Such dephosphorylation of Ser¹⁰ H3 is unusual, because most stimuli increase phosphorylation at this residue. To date, only three stimuli have been reported to induce a decrease in phospho-Ser¹⁰ H3: heat shock, an increase in intracellular calcium, and the SP600125 compound. SP600125, a JNK inhibitor, reduces global Ser¹⁰ H3 phosphorylation independently of JNK and through an unknown mechanism (26), whereas intracellular calcium and heat shock reduced global Ser¹⁰ H3 phosphorylation through the phosphatase PP2A (27, 28). Although LLO induces an influx of calcium into host cells, neither blocking this influx with EGTA or BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid] nor inhibiting PP2A with the strong PP2A inhibitor, okadaic acid (data not shown), prevented dephosphorylation of Ser¹⁰ H3 by LLO. Therefore, the signaling cascade activated by LLO seems to induce dephosphorylation of Ser¹⁰ H3 through a phosphatase not yet described as having a role in histone modifications. Furthermore, blocking other serine/threonine phosphatases, such as PP1 or PP2B, with okadaic acid (see above) and cypermetrin, respectively, also had no effect on LLO-induced dephosphorylation (data not shown), suggesting that these major cellular phosphatases are not involved in this effect. Alternatively, our results do not exclude that LLO could be inhibiting the activity of a kinase active in resting cells.

Close association of phospho-Ser¹⁰ H3 with actively transcribed genes has implicated this modification in transcriptional regulation. However, the precise role of this modification in transcription is not fully understood. It has been suggested that, in a mechanism similar to histone acetylation, where both acetyltransferases and deacetyltransferases interact with components of the transcription machinery, specific protein kinases and phosphatases might link phospho-Ser¹⁰ H3 to transcriptional regulation (26, 28). Our ChIP results indicate that a decrease in phospho-Ser¹⁰ H3 correlates with a decrease in acH4 and down-regulation of basal transcription of specific genes, whereas an increase correlates with an increase in acetylated H4 and transcriptional activation. These results support the hypothesis that the kinase/phosphatase pair of proteins modifying Ser¹⁰ induces recruitment of key factors central in transcriptional regulation. Whether modifications of Ser¹⁰ directly recruit transcription factors or are simply a prerequisite for further modifications, such as acetylation, remains to be established (reviewed in ref. 29).

Comparative studies of the eukaryotic genes whose transcription is modulated during infection have revealed a common set of genes that appear to constitute a general "alarm system" for infection (8). During a *L. monocytogenes* infection, invasion of macrophages activates cytosolic pathogen surveillance, including expression of many genes regulated by IFN- β (9). Furthermore, invasion of endothelial cells and escape from the vacuole into the host cytoplasm was recently reported to induce phosphorylation of H3 and acetylation of H4 at the promoters of several cytokines (30). Therefore, epigenetic regulation by extracellular *L. monocytogenes*, through LLO, could target the immune response in anticipation of its later activation. Indeed, a number of genes down-regulated by LLO are involved in immune responses, reflecting a mechanism by which *L. monocytogenes* is counteracting host immunity before invasion. This hypothesis is supported by the findings that *L. monocytogenes* is a bacterial pathogen that causes little inflammation during infection. In agreement with this property, we have shown that LLO specifically represses transcription of *cxcl2*, which encodes a chemoattractant chemokine with proinflammatory func-

tion. Down-regulation of CXCL2 could reduce the recruitment of polymorphonuclear cells, key players in the innate immune response and the clearing of *L. monocytogenes* during infection (31). In contrast, CXCL2 is highly up-regulated in response to infection by the very inflammatory intracellular pathogen *Shigella flexneri* (32), and down-regulation of CXCL2 expression, among other cytokines, by preinfection with a commensal *Lactobacillus casei* attenuates the proinflammatory signaling induced by *S. flexneri* (33). Another down-regulated gene identified in our study is *dusp4*, a phosphatase important for regulating MAPK signaling. The phosphatase DUSP4 (or MKP2) is usually up-regulated on activation of the MAPK pathway as a feedback mechanism. Its down-regulation on addition of LLO might suggest down-regulation of this pathway, a mechanism commonly used by many bacteria during infection to reduce the inflammatory response (reviewed in ref. 34). LLO also was found to down-regulate IFN regulatory factor-3 (*irf-3*), which encodes the major IFN transcription factor, and a common target during viral infections (ref. 35 and references therein). Additionally, transcription of the early growth response 1 (*EGRI*) gene, encoding a transcription factor important for normal development and functioning of the immune system (36) was down-regulated by LLO. In striking contrast, none of the genes up-regulated by LLO has known implications in the immune response.

An important finding in this study is that histone modifications are not merely induced by LLO of *L. monocytogenes*, but other toxins of the CDC family, e.g., *C. perfringens* PFO and *S. pneumoniae* PLY, also induce the same dephosphorylation of Ser¹⁰ H3. Interestingly, the two corresponding bacterial species are unrelated to *L. monocytogenes* and extracellular pathogens, supporting our finding that, in the case of LLO, epigenetic regulation is induced from the outside of the cell. Interestingly, several previous studies support our hypothesis that epigenetic regulation by PFO and PLY leads to suppression of the inflammatory response. First, it has long been known that infection by *C. perfringens* is remarkable for the absence of inflammatory cells. In an attempt to explain this lack of inflammation, the secretion of the proinflammatory cytokine IL-8 by endothelial cells incubated with PFO was measured and shows a decrease compared with an unstimulated control (37). A similar result is observed during a *S. pneumoniae* infection. Indeed, a Δ ply mutant induces a greater inflammatory response *in vivo* than a wild-type strain (38). Therefore, the marked decrease in phosphorylated Ser¹⁰ H3 that we report here could explain the mechanism by which these toxins are influencing the host immune response.

The mechanism of immune subversion through epigenetic modulation is an emerging concept in the study of pathogenic and commensal bacteria, although the mechanisms used appear to differ. In our study, we report a global dephosphorylation of H3 by the CDC family of toxins, correlating with repression of host immunity genes. The few previously reported histone modifications induced by bacteria differ in that, rather than decreasing immunity gene expression as we demonstrate here, other bacteria block their activation. The 19-kDa protein of mycobacteria was shown to block IFN- γ -induced histone acetylation and gene expression (39, 40). Very recently, OspF of *Shigella* was shown to block MAPK-induced H3 phosphorylation, thereby preventing activation of a subset of NF- κ B-regulated genes (41). Interestingly, commensals have the potential to induce an inflammatory response *in vivo* in intestinal epithelial cells; however, a complex network of antiinflammatory signals, which results in blocking acetylation/phosphorylation of histone H3 at the promoter of the key immune gene, *IL-6*, prevents such a response and maintains intestinal homeostasis (42). Therefore, although different mechanisms are exploited, manipulating host immunity and/or responses to invading pathogens by affecting host transcription through modification of histones appears as a common mechanism used by bacteria to downplay the host cellular response to their own advantage.

Materials and Methods

Bacterial Growth Conditions. *L. monocytogenes* strains were grown in brain–heart infusion medium (Difco, Detroit, MI) at 37°C. When required, chloramphenicol was added at 7 µg/ml.

Cell Culture and Infections. HeLa cells were grown to semiconfluence in MEM–glutamax (Invitrogen, Carlsbad, CA) supplemented with 10% FCS. Cells were then serum-starved (0.25% serum) for 24 h before use in experiments. Exponential-phase bacteria were washed twice in PBS and added to HeLa cells at a multiplicity of infection of 100. After 1 h of infection, HeLa cells were washed and 10 µg/ml gentamicin was added.

Cell Extraction, Immunoblotting, and LLO Purification. Total cell lysates from a six-well tissue culture dish were harvested by removing growth medium, adding 100 µl of PBS and 20 µl of 5× lysis buffer [0.3 M Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.05% bromophenol blue, 1.5% DTT]. Samples are then sonicated for 3 s, boiled for 5–10 min, and loaded on a 15% acrylamide gel. Transfer was done in semidry conditions (1 h at 32 mA per transfer) and blocked in 10% milk. Primary antibodies used and dilutions are as follows: 1:2,500 phospho-Ser¹⁰ H3 (05-817; Upstate, Lake Placid, NY), 1:5,000 H3 (ab1791; Abcam, Cambridge, MA), 1:2,500 acH4 (06-866; Upstate), H4 (ab10158; Abcam), AcH3 (polyclonal 06-599; Upstate), monoclonal acetyl K9H3 (ob 4441; Abcam), acetyl K14H3 (07-353; Upstate), trimethyl K9 (ob 8898, Abcam), dimethyl K9 (gift from Thomas Jenuwein, Research Institute for Molecular Pathology, Vienna, Austria) and actin (A5441; Sigma, St. Louis, MO). HRP-conjugated secondary antibodies from AbCys (Paris, France) were used at 1:10,000 dilution. Immunoblots were revealed by using ECL detection kit from Pierce (Rockford, IL). Quantification of Western blots was performed on scanned images. NIH Image was used to determine band intensities, which were then normalized to actin and to untreated cells. LLO was purified as in ref. 43.

Microarray Analysis. Total RNA from untreated and treated cells (6 nM LLO for 20 min) was extracted and purified by using the RNeasy kit (Qiagen, Valencia, CA). Quality of RNAs and cRNAs was monitored on Agilent RNA Nano LabChips (Agilent Tech-

nologies, Palo Alto, CA). RT on 5 µg of total RNA using oligo(dT) primers and *in vitro* transcription of the cDNA in presence of biotin were performed by using a GeneChip Amplification One-Cycle Target Labeling kit according to Affymetrix (Santa Clara, CA) standard protocols. Fragmented, biotin-labeled cRNA samples were hybridized on Array Type GeneChip Human Genome U133 Plus 2.0. For each condition, three biological replicates were hybridized. The cell intensity files were generated with GeneChip Operating Software (GCOS). Data analysis was performed by using SPlus ArrayAnalyser software (Insightful, Seattle, WA). Data processing was done with the GC–robust multiarray analysis method (44). Statistical analysis to compare experimental condition versus control condition was done by using the Local Pool error test (45). The *P* values (the probability that the variability in a gene behavior observed between classes could occur by chance) were adjusted by using the Benjamini–Hochberg algorithm (46).

RT-PCR. RNA from was extracted by using the RNeasy (Qiagen) kit. RT was performed by using the Invitrogen SuperScript III First-Strand Synthesis kit with random hexamers. qPCR was performed by using the SYBR Green kit and analyzed by using the MxPro software (Stratagene, La Jolla, CA).

ChIP. ChIP and qPCR were performed as described (47); briefly, after LLO treatment, HeLa cells were formaldehyde-fixed, extracted, sonicated, and then immunoprecipitated. Immunoprecipitated DNA was analyzed by qPCR, normalized to the amount of chromatin immunoprecipitated with IgG and to the amount of chromatin associated to H3.

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- Hamon M, Bierne H, Cossart P (2006) *Nat Rev Microbiol* 4:423–434.
- Tweten RK (2005) *Infect Immun* 73:6199–6209.
- Villanueva MS, Sijts AJ, Pamer EG (1995) *J Immunol* 155:5227–5233.
- Tang P, Rosenshine I, Cossart P, Finlay BB (1996) *Infect Immun* 64:2359–2361.
- Repp H, Pamukci Z, Koschinski A, Domann E, Darji A, Birringer J, Brockmeier D, Chakraborty T, Dreyer F (2002) *Cell Microbiol* 4:483–491.
- Drams S, Cossart P (2003) *Infect Immun* 71:3614–3618.
- Kayal S, Lilienbaum A, Poyart C, Memet S, Israel A, Berche P (1999) *Mol Microbiol* 31:1709–1722.
- Jenner RG, Young RA (2005) *Nat Rev Microbiol* 3:281–294.
- McCaffrey RL, Fawcett P, O'Riordan M, Lee KD, Havell EA, Brown PO, Portnoy DA (2004) *Proc Natl Acad Sci USA* 101:11386–11391.
- Strahl BD, Allis CD (2000) *Nature* 403:41–45.
- Clayton AL, Mahadevan LC (2003) *FEBS Lett* 546:51–58.
- Saccani S, Pantano S, Natoli G (2002) *Nat Immunol* 3:69–75.
- Brownell JE, Zhou J, Ranalli T, Kobayashi R, Edmondson DG, Roth SY, Allis CD (1996) *Cell* 84:843–851.
- Mahadevan LC, Willis AC, Barratt MJ (1991) *Cell* 65:775–783.
- Lieberman PM (2006) *Trends Microbiol* 14:132–140.
- Gaillard JL, Berche P, Mounier J, Richard S, Sansonetti P (1987) *Infect Immun* 55:2822–2829.
- Jacobs T, Darji A, Frahm N, Rohde M, Wehland J, Chakraborty T, Weiss S (1998) *Mol Microbiol* 28:1081–1089.
- Nato F, Reich K, Lhopital S, Rouyre S, Geoffroy C, Mazie JC, Cossart P (1991) *Infect Immun* 59:4641–4646.
- Gilbert RJ, Jimenez JL, Chen S, Tickle IJ, Rossjohn J, Parker M, Andrew PW, Saibil HR (1999) *Cell* 97:647–655.
- Jacobs T, Cima-Cabal MD, Darji A, Mendez FJ, Vazquez F, Jacobs AA, Shimada Y, Ohno-Iwashita Y, Weiss S, de los Toyos JR (1999) *FEBS Lett* 459:463–466.
- Michel E, Reich KA, Favier R, Berche P, Cossart P (1990) *Mol Microbiol* 4:2167–2178.
- Wadsworth SJ, Goldfine H (1999) *Infect Immun* 67:1770–1778.
- Thomson S, Mahadevan LC, Clayton AL (1999) *Semin Cell Dev Biol* 10:205–214.
- Chen LF, Greene WC (2004) *Nat Rev Mol Cell Biol* 5:392–401.
- Mellstrom B, Naranjo JR (2001) *Curr Opin Neurobiol* 11:312–319.
- Huang W, Batra S, Korrapati S, Mishra V, Mehta KD (2006) *Mol Cell Biol* 26:1307–1317.
- Nowak SJ, Pai CY, Corces VG (2003) *Mol Cell Biol* 23:6129–6138.
- Huang W, Batra S, Atkins BA, Mishra V, Mehta KD (2005) *J Cell Physiol* 205:37–46.
- Nowak SJ, Corces VG (2004) *Trends Genet* 20:214–220.
- Schmeck B, Beermann W, van Laak V, Zahlten J, Opitz B, Wizenrath M, Hocke AC, Chakraborty T, Kracht M, Rosseau S, et al. (2005) *J Immunol* 175:2843–2850.
- Pamer EG (2004) *Nat Rev Immunol* 4:812–823.
- Pedron T, Thibault C, Sansonetti PJ (2003) *J Biol Chem* 278:33878–33886.
- Tien MT, Girardin SE, Regnault B, Le Bourhis L, Dillies MA, Coppee JY, Bourdet-Sicard R, Sansonetti PJ, Pedron T (2006) *J Immunol* 176:1228–1237.
- Espinosa A, Alfano JR (2004) *Cell Microbiol* 6:1027–1040.
- Melroe GT, Silva L, Schaffer PA, Knipe DM (2007) *Virology* 360:305–321.
- McMahon SB, Monroe JG (1996) *J Leukoc Biol* 60:159–166.
- Bryant AE, Stevens DL (1996) *Infect Immun* 64:358–362.
- Benton KA, Everson MP, Briles DE (1995) *Infect Immun* 63:448–455.
- Pennini ME, Pai RK, Schultz DC, Boom WH, Harding CV (2006) *J Immunol* 176:4323–4330.
- Wang Y, Curry HM, Zwilling BS, Lafuse WP (2005) *J Immunol* 174:5687–5694.
- Arbibe L, Kim DW, Batsche E, Pedron T, Mateescu B, Muchardt C, Parsot C, Sansonetti PJ (2007) *Nat Immunol* 8:47–56.
- Haller D, Holt L, Kim SC, Schwabe RF, Sartor RB, Jobin C (2003) *J Biol Chem* 278:23851–23860.
- Glomski II, Decatur AL, Portnoy DA (2003) *Infect Immun* 71:6754–6765.
- Wu Z, Irizarry R, Gentleman R, Murillo F, Spencer F (2004) *J Am Stat Assoc* 99:909–917.
- Jain N, Thattai J, Braciale T, Ley K, O'Connell M, Lee JK (2003) *Bioinformatics* 19:1945–1951.
- Benjamini Y, Hochberg Y (1995) *Controlling the False Discovery Rate* (Blackwell, Oxford).
- Batsche E, Yaniv M, Muchardt C (2006) *Nat Struct Mol Biol* 13:22–29.
- Cossart P, Vicente MF, Mengaud J, Baquero F, Perez-Diaz JC, Boche P (1989) *Infect Immun* 57:3629–3636.
- Lecuit M, Sannenburg JL, Cossart P, Gordon JI (2007) *J Biol Chem* 282:15065–15072.